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## **Feline infectious peritonitis: still an enigma?**

Kipar, A ; Meli, M L

**Abstract:** Feline infectious peritonitis (FIP) is one of the most important fatal infectious diseases of cats, the pathogenesis of which has not yet been fully revealed. The present review focuses on the biology of feline coronavirus (FCoV) infection and the pathogenesis and pathological features of FIP. Recent studies have revealed functions of many viral proteins, differing receptor specificity for type I and type II FCoV, and genomic differences between feline enteric coronaviruses (FECVs) and FIP viruses (FIPVs). FECV and FIP also exhibit functional differences, since FECVs replicate mainly in intestinal epithelium and are shed in feces, and FIPVs replicate efficiently in monocytes and induce systemic disease. Thus, key events in the pathogenesis of FIP are systemic infection with FIPV, effective and sustainable viral replication in monocytes, and activation of infected monocytes. The host's genetics and immune system also play important roles. It is the activation of monocytes and macrophages that directly leads to the pathologic features of FIP, including vasculitis, body cavity effusions, and fibrinous and granulomatous inflammatory lesions. Advances have been made in the clinical diagnosis of FIP, based on the clinical pathologic findings, serologic testing, and detection of virus using molecular (polymerase chain reaction) or antibody-based methods. Nevertheless, the clinical diagnosis remains challenging in particular in the dry form of FIP, which is partly due to the incomplete understanding of infection biology and pathogenesis in FIP. So, while much progress has been made, many aspects of FIP pathogenesis still remain an enigma.

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**Review for the Special Issue of *Veterinary Pathology* on the topic of infectious diseases**

**Feline Infectious Peritonitis, still an enigma?**

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## **Abstract**

Feline infectious peritonitis (FIP) is currently the most relevant fatal infectious disease of cats. Despite intensive research in the field, its pathogenesis is still not completely revealed. FIP is a challenging clinical disease, with progressively worsening general symptoms, i.e. fluctuating fever, anorexia, and weight loss, in combination with a variety of additional clinical features that reflect the type and distribution of the pathological changes. Several, also very recent reviews, have been published that thoroughly cover all relevant clinical aspects of the disease.<sup>41,71,131</sup> In the present review, we therefore focus on the pathological features of FCoV infection and FIP and the various aspects that are relevant for the pathogenesis of the disease.

**Keywords:** Diagnosis, Feline Coronavirus, Feline infectious peritonitis, Feline infectious peritonitis virus, Feline enteric coronavirus, pathogenesis

## History

Feline infectious peritonitis (FIP) has first been thoroughly described and named in 1966, when experimental infections of healthy cats with organ material of diseased animals confirmed it as a specific, fatal infectious disease of cats, and a viral etiology was suspected.<sup>172</sup> However, the disease syndrome had already been observed in the 1950s and 60s in the USA, and even earlier, a very similar disease had been reported from cats in Naples.<sup>17,83</sup>

In 1968, the viral etiology was demonstrated.<sup>165</sup> The virus morphology suggested a coronavirus (CoV), which was finally confirmed in 1976.<sup>116,121,167</sup> The virus was first grown in peritoneal cells of experimentally infected cats, and, after propagation in cell culture, was shown to cause FIP in 100% of intraperitoneally infected animals.<sup>123</sup> Subsequently, the macrophage cell line *Felis catus* whole fetus-4 (Fcwf-4) has predominantly been used for virus propagation.<sup>87,123</sup>

In his recent review article, based on more than 40 years of work in the field, Pedersen speculated on the reason for the likely emergence of FIP in the 20<sup>th</sup> century. He considered as the most relevant potential factors the evolution of the feline CoV (FCoV) alongside CoV of pigs and dogs, the development of virulent FIP virus mutations from enteric FCoV that had only evolved at that stage, and the changes in keeping and especially breeding of cats due to their increasing popularity as pets.<sup>131</sup>

At present, despite decades of research on its etiology, pathogenesis, transmission and prevention, FIP is still the most frequent fatal and infectious feline disease for which there is so far no effective cure.

## Feline Coronaviruses (FCoV)

63    *Virology*

64    FCoV are pleiomorphic enveloped, single-stranded positive sense RNA viruses with  
65    an almost 30kb non-segmented genome and 11 putative open reading frames  
66    (ORF). They belong to the family *Coronaviridae*, order *Nidovirales*, and, together with  
67    Canine Coronavirus (CCV) and Transmissible Gastroenteritis Virus (TEGV) of pigs,  
68    belong to the subfamily *Coronavirinae*, genus *Alphacoronavirus*, species  
69    *Alphacoronavirus 1*.<sup>61</sup>

70    At the 5' end of the FCoV genome, approximately 20kb comprise the two overlapping  
71    ORF 1a and 1b that encode for two polypeptides which are subsequently  
72    enzymatically cleaved into 16 non-structural functional proteins mainly involved in the  
73    synthesis of the viral RNA (viral replicase). The remaining genome contains nine  
74    ORF that encode for four structural proteins (spike [S], nucleocapsid [N], membrane  
75    [M], and envelope [E]) and five group-specific, accessory proteins (3a-c, 7a and b).  
76    These are expressed individually from a nested set of subgenomic mRNAs that each  
77    contain a leader RNA sequence derived from the 5' end of the genome, and are  
78    generated by discontinuous transcription from the 3' end of the genome.<sup>44,46</sup> The CoV  
79    envelope is formed by the S protein, a 180-200 kDa glycoprotein arranged in  
80    peplomers that induces the antibody response and cell mediated immunity in the  
81    host. The S peplomers are 12-24 nm long, dome shaped, and arranged like a crown;  
82    they are the key determinants of cell tropism.<sup>13</sup> The S protein is a type I  
83    transmembrane protein with a very short C-terminal cytoplasmic tail and a long N-  
84    terminal ectodomain that is divided into a N-terminal (S1) domain responsible for  
85    receptor binding, and a C-terminal (S2) domain containing the fusion peptide which  
86    mediates fusion with the target cell membrane.<sup>18</sup> The M and E proteins are smaller  
87    surface glycoproteins and important for virus maturation, assembly, budding and

88 interaction with the host cell. The M protein, with a mass of approximately 29 kDa,  
89 penetrates the envelope and connects it to the capsid, and participates in the RNA  
90 packaging. E proteins are type III membrane proteins of about 9 kDa that interact  
91 with the M protein in the budding compartment of the host cell.<sup>44</sup> In mouse hepatitis  
92 virus (MHV), they can induce apoptosis.<sup>7</sup> The N proteins have a molecular weight of  
93 approximately 50 kDa. Together with the viral RNA, they form the flexible, helical  
94 nucleocapsid and seem to be critical for viral transcription.<sup>114</sup> Vaccine studies based  
95 on the N protein indicate that it induces cell mediated immunity and can play a  
96 protective role.<sup>82</sup> So far, no specific function could be ascribed to the accessory  
97 proteins. The 71-72 amino acid long 3a and b proteins are well conserved among  
98 subspecies 1 alphacoronaviruses. Since they lack predicted hydrophobic segments,  
99 both are thought to be located and exert their function in the cytoplasm. ORF 3c is  
100 very well conserved among the alphacoronavirus genus and its predicted sequence  
101 indicates that it is a class III triple spanning membrane protein of 238-244 residues,  
102 with a topology similar to that of the M protein.<sup>68,115</sup> ORF 7a encodes for a small  
103 membrane protein of approximately 10 kDa with N-terminal cleavable signal  
104 sequence and a C-terminal transmembrane domain. A recent study with deletion  
105 mutants of the homologous TGEV protein has found evidence that 7a impairs the  
106 host's antiviral response.<sup>32</sup> The ORF 7b is present only in FCoV, CCV and ferret CoV  
107 and encodes for a soluble glycoprotein of 207 residues (approximately 24 kDa) that  
108 has been shown to induce antibodies in naturally infected cats.<sup>76,90</sup>

109 CoV occur in many mammalian species including humans, and in birds. They lead to  
110 acute or chronic infections and, depending on their cell tropism, induce highly  
111 variable diseases in their hosts. The host and tissue specificity is dependent on  
112 sequence variations of the S gene as well as receptor usage and distribution.<sup>46</sup>

RNA viruses have high error rates in their replication and therefore occur as quasispecies, i.e. groups of related genotypes.<sup>37</sup> With every RNA replication of CoV, several point mutations occur. Even virus “stocks” prepared from plaques form quasispecies. Genetic diversity within a quasispecies has been suggested to contribute to pathogenesis by cooperative interactions among variant viruses within a population.<sup>163</sup> On the other hand, proofreading or repair mechanisms, mediated by the exoribonuclease in the replicase complex, allow RNA viruses to evolve whilst keeping a balance between adaptation and viral fitness.<sup>37</sup> Also, homologous RNA recombination during mixed infections of closely related CoV strains in the same group promote cross-species transmission and pathogenesis; the cat might represent a “mixing vessel” as *in vitro* studies showed that feline aminopeptidase N can be used as a functional receptor by closely related alphacoronaviruses, such as FCoV, CCV, TGEV, and human coronavirus HCoV-229E.<sup>158</sup>

Genome sequences and subsequent phylogenetic analysis showed that FCoV isolates form geographical clusters.<sup>10-12,42,102,132</sup> FCoV from cats of the same household exhibit over 95% genetic identity, suggesting infection from a common virus.<sup>5,102,162</sup> Focussing on the S gene, one study specifically examined the evolution of virus strains in cohorts of naturally infected cats over several years. It demonstrated very high conservation of the virus in persistently infected and (recurrently) shedding animals, but also showed that cats can become transiently infected and subsequently re-infected with the same or a different strain. There was also evidence of super- or co-infection of persistently infected cats with other strains.<sup>5</sup>

## Serotypes

As shown by virus neutralizing antibody reaction and amino acid sequences of the S protein, FCoV form two antigenetically distinct serotypes, type I FCoV which are difficult to grow in cell culture, and type II FCoV which are the consequence of a double recombination between type I FCoV and CCV.<sup>79,112,127,143</sup> *In vitro*, the growth kinetics of both serotypes appear to be solely related to the S protein, as determined using a recombinant type I FCoV encoding a type II S protein.<sup>157</sup> For type II FCoV, like for several other alphacoronaviruses, the cell receptor is aminopeptidase N (APN, CD13) which upon binding to the S protein, mediates the viral internalization into the target cells.<sup>81,157</sup> Antibody blockage of APN has been shown to severely reduce the infection of bone marrow derived macrophages (BMDM) with the type II FIP strain 79-1146.<sup>140</sup> However, it has so far not been confirmed that APN is also the receptor for FIPV II in infected animals. Furthermore, the receptor for serotype I FCoV is not known. Interestingly, however, isolated feline monocytes rapidly internalize both serotype I and II FIPV and accumulate the virus particles in endosomes, followed by particle disassembly.<sup>160</sup> Both serotypes can use dendritic cell (DC)-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN, CD209), a C-type lectin, which recognizes high-mannose oligosaccharides as ligands, to infect monocyte-derived dendritic cells.<sup>138</sup> Co-localization and binding inhibition studies confirmed that DC-SIGN and not APN is involved in the entry process of serotype I FCoV in monocytes, whereas for serotype II FCoV, both APN and DC-SIGN play a role in infection of monocytes, i.e. binding is mediated by APN but DC-SIGN is important for either internalization or a subsequent step.<sup>161</sup> In both models, a role of an unknown co-receptor cannot be excluded.

Both FCoV serotypes can cause FIP, but serological and, more recently, molecular studies confirmed that type I FCoV dominate by far in the cat population worldwide,



with a prevalence of up to 98%.<sup>5,80,105,107,127,143</sup> Type I FCoV were shown to induce higher antibody titres than type II FCoV, and were more frequently associated with clinical signs and/or FIP.<sup>105</sup> A higher type II prevalence, partly together with type I infection, has been reported for cats with FIP, ranging from 10% to more than 30%, the latter in an older study in Japan.<sup>15,42,107,143</sup>

### *Feline Enteric Coronavirus versus Feline Infectious Peritonitis Virus*

FCoV occur as two pathotypes, Feline Enteric Coronavirus (FECV), defined as the “ubiquitous enteric biotype”, and Feline Infectious Peritonitis Virus (FIPV), the “virulent biotype that causes FIP in individual cats”.<sup>131</sup> FECV and FIPV cannot be distinguished serologically or morphologically, and for many years, the search for markers that can discern the two pathotypes remained unsuccessful. In the past, it was assumed that the main difference between FECV and FIPV was that FECV exclusively infect the intestinal epithelium and do not pass the intestinal mucosal barrier, while FIPV infect and replicate in monocytes/macrophages and can therefore gain access to the blood and induce the disease.<sup>124,125,128</sup> When more sensitive molecular methods became available, this hypothesis was proven too simple. It was shown that also FECV can infect monocytes and that FCoV generally spread from the initial site of infection, the intestine, via monocyte-associated viremia.<sup>62,94,98,109</sup> Indeed, approximately 80% of cats, healthy or with FIP, in households with endemic FCoV infection were shown to harbor FCoV RNA in their blood monocytes, and healthy cats remained viremic over the 12 month test period.<sup>62</sup> Furthermore, it was shown recently that intraperitoneal inoculation with FECV can, albeit only occasionally, lead to virus shedding with the feces, which confirms FECV

spread also from extraintestinal sites, again most likely via monocyte-associated viremia.<sup>133</sup>

In order to test whether the difference between FECV and FIPV is based on the exclusive capability of FIPV to replicate in feline monocytes, a new method was developed that demonstrates replicating virus in the blood, through the specific detection of viral M protein mRNA.<sup>145</sup> The protocol was applied in two studies on naturally infected cats and showed that FCoV can replicate within monocytes in healthy cats. However, while the first study found a strong correlation between virus replication in the blood and FIP, the second did not confirm this finding.<sup>24,145</sup>

Subsequently, a quantitative method detected high levels of viral replication in cats suffering from FIP.<sup>84</sup> Furthermore, a recent experimental study showed that after oronasal infection with known FECV isolates, only very few cats develop viremia, and without evidence of viral replication.<sup>164</sup>

Initial molecular studies on FECV and FIPV isolates identified deletions in the 3c, 7a and 7b genes in FIPV and indicated that FECV are the ancestors of FIPV.<sup>89,162</sup> Also, deletion of the 3a-c and/or 7a/7b genes from wild type FIPV II (79-1146) in an attempt to generate FIP vaccines led to the loss of virulence in experimental infections.<sup>67</sup> This initial work was followed by a more rigorous search for virulence markers. The sequencing of structural (S, E, M, N) and accessory (3a-c, 7a and b) genes of FCoV from feces and diseased tissues of cats with FIP identified significant mutations only in the 3c gene; these resulted in variable truncation of the 3c protein. Virus with the mutated 3c gene was identified in diseased tissues, whereas the FCoV in the feces generally exhibited an intact 3c gene, and only in some cases also the mutated form.<sup>132</sup> Together with the results of a previous study, this indicated a role of 3c gene deletions in the viral switch from FECV to FIPV.<sup>132,162</sup> However, the fact that

3c gene deletions were not consistently observed with FIP suggested that additional factors are essential for the acquisition of the FIPV pathotype.<sup>162</sup>

Two larger subsequent studies compared the 3c gene of FCoV from healthy cats (FECV) and cats with FIP (FIPV) to further assess its role as a virulence marker.

Almost all FECV carried an intact 3c gene.<sup>28,133</sup> After oronasal inoculation with such FECV isolates, cats became infected and shed the virus with the feces.<sup>133</sup> In contrast, the majority (71%) of FCoV from cats with FIP exhibited 3c mutations, i.e. deletions and insertions that were sometimes associated with severe truncation and loss of function. Interestingly, FCoV identified in the feces of cats with FIP generally exhibited an intact 3c gene, which was interpreted as an indication of FECV superinfection.<sup>26</sup> Nonetheless, similar to a previous study, both these studies identified FIPV with an intact 3c gene in diseased tissues in a substantial proportion of cats with FIP (29% and 40% respectively).<sup>26,133,162</sup> Furthermore, the FCoV with an intact 3c gene were shown to be indeed FIPV, since they induced FIP after both oronasal and intraperitoneal inoculation.<sup>133</sup> Interestingly, when shed with the feces, the latter was not infectious to cats.<sup>133</sup> These findings lead to the following conclusion: FCoV need to carry an intact 3c gene to be able to sustainably replicate in the intestinal epithelium and be infective to other cats. However, an intact/non-truncated 3c gene does not prevent a FCoV from inducing FIP even after oronasal inoculation. The potential significance of the observed higher frequency of non-synonymous amino acid changes towards the 3' end of FIPV with intact 3c genes needs to be further investigated.<sup>133</sup>

A non-targeted approach has recently been taken in an attempt to identify further mutations that might be responsible for the change in virulence. Each 11 randomly selected FECV (from healthy cats) and FIPV (from cats with FIP confirmed by post

mortem examination) were compared, based on full genome sequencing. Differences were found scattered along the entire genome, but a larger genetic variation with two hot spots was identified in the S gene. Subsequent sequencing and phylogenetic analysis of more isolates identified the two alternative codons in the S gene in more than 95% of the examined FIP cases. Both mutations occur in the supposed fusion peptide of the S protein, but without any evidence of potential functional consequences.<sup>28</sup> However, due to the mutation rate of RNA viruses and the relative rarity of FIP, the authors concluded that the identified S gene mutations are unlikely solely responsible for the FECV-FIPV virulence switch.<sup>28</sup>

A group that has worked extensively on antibody mediated enhancement (ADE) in FIP used FCoV that are resistant to virus neutralizing monoclonal antibodies to search for virulence markers. Their so-called mar-mutant viruses all exhibit mutations in several amino acids in the S1 region.<sup>91</sup> However, when orally administered to cats, only some were found to induce FIP. None of the latter carried mutations in ORF 2-7 other than those observed in the S1 region, whereas the avirulent viruses also showed deletions in the 7b gene.<sup>154</sup>

A further molecular study on natural cases identified a relatively high diversity of the N protein in endemically infected cat groups, but without any pattern or relation to virulence.<sup>12</sup> Furthermore, analysis of nucleotide substitutions identified residues in the N protein that were subjected to positive selection. These could represent antigenic immunodominant sites, indicating the antigenic role of the N protein in stimulating cell-mediated immunity.<sup>12</sup>

*In vitro* studies complement the *in vivo* approaches and provide strong evidence that FCoV virulence requires the ability to productively and sustainably infect feline monocytes. This was first indicated in an older study which demonstrated less

261 effective and shorter replication of avirulent FCoV than FIPV in feline peritoneal  
262 macrophages and was more recently confirmed in isolated feline monocytes, in  
263 which a FIPV (79-1146) established sustainable replication, whereas a FECV (79-  
264 1683) could replicate, but not sustainably.<sup>38,149</sup> It needs to be emphasized though that  
265 despite rapid virus binding, internalization and disassembly, even the replication of  
266 FIPV is limited to a very small proportion of macrophages and monocytes.<sup>38,149,160</sup>  
267 This strongly suggests that most monocytes/macrophages are resistant to the virus  
268 at the time of infection, most likely due to inhibition of genome release and/or  
269 translation.<sup>160</sup>

270 Attempts have been made to relate the monocyte/macrophage tropism to differences  
271 in the viral protein structure. In BMDM cultures, FECV (79-1683) was shown to infect  
272 fewer cells than FIPV (79-1146) and appeared unable to spread the infection.<sup>140</sup> This  
273 was determined by the S protein alone, and, interestingly, by the membrane-proximal  
274 S2 domain involved in virus mediated membrane fusion, and not the receptor binding  
275 S1 region.<sup>140</sup> In agreement with these findings, a recent study found that deletions in  
276 the S1 gene region did not affect the viral capacity to productively infect feline  
277 monocytes. However, a N-terminal 29 amino acid deletion in the 7b gene lead to a  
278 decrease in virulence, although one of these mutants still retained the capacity to  
279 productively infect macrophages, suggesting that this ability is not mediated by ORF  
280 7b.<sup>154</sup>

281 Full sequencing of the FIPV type II strain DF2 has shown that it carries a 338-nt  
282 deletion in the ORF3abc, resulting in the truncation of 3a and 3c and the complete  
283 loss of ORF3b. This virus replicated efficiently in isolated feline monocytes. When the  
284 DF2 ORF3abc was replaced with a genetically closely related, intact CCV ORF3abc  
285 region, the recombinant virus was able to replicate in feline monocytes, but yielded

significantly lower virus titers.<sup>9</sup> These results are in contrast to those of an investigation into the relevance of the ORF3 and 7 proteins for the replication of FCoV in monocytes published a year later, using the type II FIPV 79-1146, which is thought to obtain its virulence through its S protein structure but has also been shown to have a truncated 3c.<sup>35,140</sup> The use of genetically modified viruses with deletion of ORF 3abc (FIPV- $\Delta$ 3), 7ab (FIPV- $\Delta$ 7) or both (FIPV- $\Delta$ 3 $\Delta$ 7) demonstrated a lower, but sustainable replication capacity in the absence of 3, whereas viruses lacking 7ab could only undergo one replication cycle.<sup>35,67</sup> Since ORF 7 are located at the 3' end of the genome where transcription begins, 7a and 7b are produced very early in viral replication; it was therefore concluded that they might neutralize the innate immune response to the virus during the early phase of infection, for example by counteracting the IFN-mediated induction of an antiviral state, resulting in inhibition of viral replication.<sup>35,44</sup> A contradiction remains to be clarified since Rottier and co-workers showed in their study that mutants lacking the 7b gene could still productively replicate in macrophages and mentioned, without showing the results, that the same applied for mutated viruses lacking both 7a and 7b.<sup>140</sup> This difference might be related to the use of different cells in both experiments, since Rottier infected BMDM, whereas the other study used peripheral blood-derived monocytes.<sup>35,140</sup>

In summary, while promising, the above results from several studies do not yet provide a conclusive picture (Table 1). This is likely also due to the general diversity of the study material, in particular with regard to the virus isolates, but also the methodological approaches that have been taken.<sup>131</sup>

## *Prevalence*

FIP is currently the leading infection cause of death in cats.<sup>131</sup> However, despite the generally high prevalence of FCoV infection in the cat population, which can exceed 90% in multicat environments, FIP morbidity is low and rarely surpasses 5% of infected cats.<sup>41,131</sup> In larger cat groups, the proportion of chronic shedders and the overall frequency of virus shedding represent risk factors.<sup>50</sup> FIP is a disease of young (6 months to 2 years), purebred, male intact cats.<sup>139</sup> Purebred cats appear to be more susceptible also to FCoV infection in general, since they were overrepresented when healthy mixed populations were screened.<sup>48,101</sup> A recent study indicates that the breed predilection is restricted. While Abyssinians, Bengals, Birman, Himalayans, Ragdolls and Rexes were found to have a significantly higher risk for the development of FIP, Burmese, Exotic Shorthairs, Manx, Persians, Russian Blues and Siamese cats did not exhibit an increased risk.<sup>134</sup>

#### *Transmission, shedding, and persistence of FCoV*

FCoV are transmitted via the fecal-oral route and primarily infect enterocytes.<sup>129</sup> Cats can become persistently infected and generally remain healthy, despite systemic infection, indicating that healthy (FECV) carriers play a key role in the epidemiology of FIP.<sup>3,4,62,77,94,100,109</sup> FCoV are shed with the feces, and carrier animals have been shown to shed the virus intermittently for months.<sup>4,49,69,78,109</sup> Furthermore, there is evidence of a correlation between shedding frequency and intensity and high antibody titres.<sup>71</sup>

Experimental studies with type I FECV isolates have demonstrated consistent shedding as early as two days and for up to 2 weeks post infection (pi), with a subsequent decline in fecal viral loads and intermittent shedding up to 20 weeks after this period.<sup>100,109,164</sup> They confirmed previous studies in which oral administration of

cell culture-adapted FIPV (Wellcome strain) led to viral antigen expression in the small and large intestine between day 1 and 7 pi, and restriction to cecum and colon on day 14.<sup>148</sup> After clearance from the small intestine, FCoV can apparently spread from the persistently infected colon at later stages, leading to renewed shedding.<sup>100</sup> In natural FIP cases, shedding can occur until death.<sup>3,147</sup> However, compared to diarrhoeic or healthy shedders, the amount of replicating virus shed by cats with FIP is only very low, and it is significantly lower in the gut than in organs.<sup>84,132</sup> Despite the generally strong evidence that only FECV and not FIPV are transmitted between cats, a recent study confirmed that the FCoV with a truncated 3c gene found in diseased tissues are occasionally also present in the feces of cats with FIP.<sup>132</sup> While this would suggest that horizontal transmission is possible, a subsequent study indicated that oronasal uptake of shed, fecal FIPV does not lead to FIP.<sup>133</sup> It remains to be clarified whether this is a universal characteristic of FIPV. This could be of relevance for epizootic FIP outbreaks, which are defined by the occurrence of FIP in more than 10% of cats in high prevalence establishments, the percentage can be lower in an environment with generally very low FIP prevalence.<sup>41,131,136</sup> In this context, a closer look into an “artificially induced” FIP outbreak that we monitored a number of years ago is of interest. As part of a trial to investigate the efficacy of a FIP vaccine, a multicat environment similar to an animal shelter was created. This housed 40 specific pathogen free (SPF) cats (20 female, 20 male neutered) to which 10 clinically healthy cats (aged 6 months to 3 years) from different animal shelters were introduced. The latter had been selected since they tested positive for circulating FCoV immune complexes.<sup>94,96,98</sup> Within one week after introduction of the shelter cats and in which fighting for the establishment of hierarchies occurred, several animals developed transient cat flu symptoms. These



subsidied, but the first FIP cases occurred in week 6 (n=4), followed by a peak in weeks 7, 8 (each n=4) and 9 (n=3), with further individual cases in weeks 14 through to 22; a total of 23 animals (45%; 22 (55%) SPF cats, 1 shelter cat) succumbed to the disease. Most SPF cats that died with FIP (18; 82%) had shown previous or concurrent flu symptoms.<sup>106</sup> Only 14 (35%) SPF cats survived the challenge period of 21 weeks. They had all become infected, had shed virus at least intermittently, and exhibited histological features in the hemolymphatic tissues that indicate a strong immune response to the virus.<sup>94,96,99</sup> The characteristics of this experimental FIP epidemic, using natural infections with field viruses, support assumptions that the occurrence of outbreaks is associated with factors related to the environment (such as crowding, concurrent infections, long-term exposure to shedders), the virus (such as virulence, replication rate and mutation rate of the strain) and the host (individual differences in the immune response to FCoV).<sup>41</sup> It also appears likely that horizontal FIPV transmission played a role in this particular case. It might indeed be the generally low amount of FIPV that is shed with the feces if at all, and the inability of at least some FIPV shed with the feces to induce FIP that prevent more frequent horizontal transmission of the disease.<sup>132,133</sup>

The main site of FCoV persistence is the colon, where viral antigen has been found in differentiated enterocytes.<sup>78,100,164</sup> However, virus can also be detected in other tissues in the absence of viremia, and has been shown to infect tissue macrophages.<sup>100</sup> Also, there is evidence of recurrent systemic spread.<sup>100</sup> These findings suggest that viremia and, ultimately, FIP can develop in infected animals at any stage after initial viremia, even when the virus is cleared from the gut.<sup>100</sup>

## **Pathologic features of FCoV infection and FIP**

### *FIP*

The name given to the disease in the 1960s acknowledges the consistent main gross pathological finding, a peritonitis (Figs. 1, 2). Upon gross post mortem examination, FIP is typically characterized by a fibrinous and granulomatous serositis, protein rich serous effusions and/or pyogranulomatous lesions in several organs (Fig. 1). The latter, however, are often very small and only identified by histological examination (Fig. 1e, f). Clinically, a rather clear distinction is made between an effusive (wet or non-parenchymatous) and a non-effusive (dry or parenchymatous) form of the disease, with a proportion of cases being considered in a transition stage between the two forms.<sup>41,71,131</sup> However, the post mortem examination often identifies extensive serosal and parenchymatous granulomatous lesions in organs alongside effusions of a variable quantity (Fig. 1a-d), indicating that mixed forms are indeed more common than clinically appreciated.

When the disease was first observed, several reports provided histopathological descriptions of both spontaneous and experimental cases.<sup>19,73,74,166,169-172</sup> Several years later, a few studies attempted to categorize FIP lesions.<sup>16,92,118</sup> Based on distribution, cellular composition, and viral antigen expression, four types of lesions were described: diffuse alterations on serosal surfaces; granulomas with and without areas of necrosis; focal and perivascular B cell and plasma cell infiltrates, and a granulomatous to necrotizing vasculitis; these can be found alongside each other.<sup>92</sup> The distribution of lesions varies in each individual case, but shows a consistent general pattern.<sup>92,96,131,172</sup> Detailed gross, histological and immunohistological examinations that we performed on a large cohort of diagnostic cases to identify all

potential lesions, confirmed peritoneal involvement in 75% of the cases, in the majority (69%) associated with abdominal effusion (Fig. 2), sometimes also with effusion in the thorax. Among organs, the kidneys were affected most often, followed by brain and eyes (Fig. 2). The latter were always involved alongside the brain, and in a subsequent study that thoroughly examined confirmed FIP cases for the presence of ocular lesions, 29% (25/86) showed involvement of the eye, in the majority of cases (68%) bilateral (M. Weber, unpublished data), suggesting that the actual involvement of the eyes is generally underestimated.<sup>131</sup> FIP lesions are occasionally seen at unusual sites, such as the tunica vaginalis in cats with peritonitis, the skin or the testicle.<sup>23,34,144</sup>

Interestingly, the general distribution of the FIP vasculitis is relatively limited. It mainly affects small and medium sized veins in leptomeninges, renal cortex (stellate veins) and eyes (mainly venules in iris, chorioidea and retina), less frequently in lungs and liver.<sup>97</sup>

For natural FIP cases, an incubation period is not known. However, we gathered some information on the time log between the onset of FCoV exposure and overt disease; when we introduced several FCoV carriers into a large group of SPF cats housed together in a shelter-like multicat environment, the first clinical signs of FIP were detected after 6 weeks.<sup>106</sup> After experimental infection, however, the incubation period has been shown to range between 2 and 14 days for the effusive and several weeks for the dry form.<sup>36,123</sup>

The clinical course of FIP in natural cases is usually quite rapid for the wet form, but can take several weeks in particular for the dry form.<sup>131</sup> We observed a clinical course of 6 to 42 days (average: 14 days) prior to death in the above mentioned group of naturally infected SPF cats.<sup>106</sup> However, evidence of subclinical or

protracted disease over a period of weeks to months has been reported.<sup>131</sup> Also, an experimental longitudinal study demonstrated recurring waves of clinical disease, where fever and weight loss coincided with T cell depletion and increased viral loads in the blood.<sup>36</sup> From such data, histological findings suggestive of “disease waves” would be expected, and indeed, these can be observed. As reported, the typical serosal FIP lesions often exhibit an underlying layer of B cells and plasma cells, and some of the latter contain FCoV-specific antibodies.<sup>92</sup> We observed occasional natural cases with serosal lesions dominated by a thick plasma cell layer and evidence of granulation tissue formation with superficial granulomas and/or a fibrinous exudate (Figs. 3, 4). There is histological evidence that in FIP granulomas, macrophages are progressively replaced by B cells and plasma cells and that the typical FIP perivascularitis can develop into the focal and perivascular B cell and plasma cell infiltrates that are frequently observed, for example, in leptomeninx and mesentery.<sup>92,97</sup> These findings indicate that the humoral immune response can limit disease progression at least to some extent or for a limited time.

The typical FIP vasculitis is a phlebitis, mediated and dominated by activated virus infected monocytes, with only few T cells and neutrophils. On the basis of these features it can be distinguished from an immune mediated vasculitis, including an immune complex vasculitis.<sup>97</sup> However, in fulminant cases, necrosis of the vessels has been seen.<sup>19,73,169</sup> Interestingly, such an acute necrotizing vasculitis can occasionally be observed in veins with apparent previous changes (Fig. 5) which further confirms the multiphasic nature of the disease. However, due to the morphological features of the acute vascular lesions, it also provides evidence that a type III hypersensitivity reaction does contribute to the pathogenesis at least in some cases.<sup>19,73,111,169,170</sup>

It has long been suspected that some cats can survive clinical FIP.<sup>126</sup> In the above-mentioned longitudinal experimental study, the majority of cats died from FIP, but several animals that had undergone one or more episodes of clinical disease survived the 4-month study period and were seen to be “free of lesions” post mortem.<sup>36</sup> Similarly, in our longitudinal study undertaken on naturally infected SPF cats, a proportion of SPF cats became infected but survived the experiment despite consistent direct contact with virus shedding carriers for 7.5 months, from the age of 21 weeks onwards.<sup>94,106,109</sup> All survivors had remained clinically healthy throughout the experiment, apart from two cats that had developed uveitis. This did not resolve in one animal which also showed transient non-specific clinical symptoms. The post mortem examination of the latter revealed a moderate chronic B cell and plasma cell dominated leptomeningitis and perivascular encephalitis as well as a severe mononuclear conjunctivitis, iridocyclitis and perineural leptomeningitis with FCoV antibody-positive plasma cells in the infiltrates (Fig. 6). These findings support the results of an older experimental vaccine study and provide further evidence that lesions can remain limited, and that macrophages in lesions can be replaced by B cells and plasma cells with time.<sup>8,126</sup>

Some animals can apparently confine the disease locally, at least for some time. One case series reported FIP lesions restricted to the mesenteric lymph nodes, and another single mural intestinal lesions, partly in association with local lymph node involvement.<sup>72,95</sup> These findings suggest a strong local response to the virus. In the latter cases, however, progression to overt FIP was generally observed despite surgical removal of the intestinal lesions.<sup>72</sup>

The lymphatic tissue of cats with FIP generally exhibits B and T cell depletion. However, in the majority of cats, this occurs with previous follicular hyperplasia and is

associated with markedly increased numbers, proliferation and activation of macrophages in the splenic red pulp, lymph node sinuses and bone marrow.<sup>92,96,99</sup>

#### *Coronavirus enteritis*

FECV is generally regarded as the avirulent pathotype of FCoV and indeed, in older cats, oral FECV infection does lead to no or only very mild, non-specific clinical symptoms, such as transient anorexia.<sup>164</sup> However, in young SPF kittens, at an age when animals would usually be protected by maternal antibodies, oral FECV infection can induce severe enteritis.<sup>1,125</sup> There have also been reports of fatal coronavirus enteritis in naturally infected juvenile and adult cats. Affected cats presented with catarrhal to hemorrhagic enteritis and immunohistology confirmed that the virus infected the fully differentiated villous epithelial cells.<sup>75,93,108</sup>

#### *Coronavirus infection without FIP*

In an environment of high infection pressure, such as a FIP outbreak, cats become FCoV-infected and develop monocyte associated viremia and a systemic immune response to the virus, as reflected by the development of antibody and circulating immune complex titres. This is associated with distinct T and B cell hyperplasia in lymphatic tissues and the presence of plasma cells expressing FCoV-specific antibodies which does not prevent virus shedding and viral spread to tissues.<sup>94,96,100,109</sup> A similar reaction of the lymphatic tissue has also been described after experimental FECV infections.<sup>66,109</sup> Interestingly, it is associated with macrophage proliferation in hemolymphatic tissues, similar to, but less intense than that seen in cats with FIP.<sup>99</sup>

511

## 512 **Pathogenesis**

513 The pathogenesis of FIP has been a research focus for several groups in Europe, the  
514 USA, and Japan. Although the picture is still not clear, the results of both *in vivo* and  
515 *in vitro* studies, though sometimes controversial, have contributed more and more  
516 pieces to the jigsaw. At present, three key features have been identified as essential  
517 prerequisites for the development of FIP lesions: systemic infection with virulent  
518 FCoV, i.e. FIPV; effective and sustainable FIPV replication in monocytes; and  
519 activation of FIPV-infected monocytes.

520

### 521 *Systemic infection with virulent FIPV*

522 Two theories have been proposed for the infection of the host; the “*in vivo* mutation  
523 transition” or “internal mutation” hypothesis and the “distinct circulating avirulent and  
524 virulent strains” hypothesis.<sup>21</sup> The first model assumes that FIPV arise *in vivo* from  
525 mutations of FECV in infected animals, and there is indeed strong evidence that the  
526 initially acquired FCoV of most cats is not a FIPV *per se*. Initial comparative genome  
527 analyses of FECV and FIPV laboratory and field strains has shown these to occur as  
528 closely related pairs.<sup>76,124,162</sup> Also, an experiment performed in chronically Feline  
529 Immunodeficiency Virus (FIV)-infected cats showed that FIPV arise *de novo* from the  
530 FECV inoculum.<sup>135</sup> Furthermore, many studies demonstrated phylogenetic clustering  
531 of FIPV and FECV according to geographic distribution rather than disease  
532 phenotype.<sup>10,27,132,135,162</sup> Finally, it is well known that although FECV are endemic in  
533 cat populations, FIP develops only sporadically, providing further strong evidence  
534 that FIPV are generally not transmitted horizontally from cat to cat, but emerge *de*  
535 *nov*o in each cat that succumbs to FIP.<sup>131</sup>

FCoV exhibit *in vivo* genetic diversity, as shown by the frequent occurrence of viral quasispecies both in individual infected animals and in infected cats from the same household.<sup>11,42,64,102</sup> Indeed, experimental infection with fecal matter of naturally FECV infected healthy cats leads to the occurrence of quasispecies in the large intestine of individual animals.<sup>102</sup> In natural infections, cats with FIP showed more extensive viral quasispecies formation than healthy animals, suggesting that a higher viral mutation rate is relevant for the generation of virulent mutants.<sup>11</sup> Surprisingly, however, recent phylogenetic analyses indicated that the observed genetic diversity mainly applies to type I FCoV, whereas type II viruses are relatively homogenous.<sup>42,107</sup> If this were indeed the case, the results of many recent studies would be called at least partly into question.

The “distinct circulating avirulent and virulent strains” hypothesis is based on phylogenetic analyses and suggests that both virulent and avirulent strains circulate in the feline population and that, independently of geographic location, sequences tend to cluster with disease phenotype.<sup>20</sup> The occurrence of occasional FIP epidemics indicates that this theory applies sporadically, while in the majority of cases, evidence supports the internal mutation theory.

#### *Effective and sustainable FIPV replication in monocytes*

The second essential prerequisite for FIP appears to be the viral capacity to replicate effectively and sustainably in monocytes of the infected host. *In vitro*, both FECV and FIPV can replicate in isolated feline peritoneal macrophages, BMDM, and monocytes, but only FIPV undergo sustainable replication and spread the infection in the culture.<sup>38,140,149</sup> These results support *in vivo* studies which have shown that FCoV infection generally leads to monocyte associated viremia, but that viral



561 replication in the blood (i.e. in monocytes) and viral loads in tissues are generally  
562 significantly higher in association with FIP.<sup>62,84,98,109,145</sup> Based on older experimental  
563 studies, Pedersen suspected that viral replication in monocytes is very slow at least  
564 during the first two weeks after FIPV infection, but then increases rapidly, around the  
565 time when specific antibodies occur.<sup>120,131,170</sup> So far, this hypothesis has not been  
566 tested in experimentally infected cats. However, it has been shown that FCoV  
567 infection of cats induces macrophage/monocyte proliferation in hemolymphatic  
568 tissues.<sup>96,99</sup> This is not associated with upregulation of cytokines that stimulate  
569 macrophage proliferation in these tissues, and could therefore represent a systemic  
570 effect of infected monocytes which we found to transcribe granulocyte-monocyte  
571 colony stimulating factor (GM-CSF) and interleukin (IL-)6, cytokines that both induce  
572 proliferation and differentiation of monocyte and neutrophil precursors, within hours  
573 after *in vitro* FIPV infection (Kipar, unpublished data). The proliferation of  
574 monocytes/macrophages likely ensures the supply of viral target cells, i.e. mature  
575 circulating monocytes or tissue macrophages. Proliferating macrophage populations,  
576 such as macrophages in the splenic red pulp or myelomonocytic cells in the bone  
577 marrow, appear not to replicate the virus, since viral antigen cannot be detected in  
578 these cells.<sup>96,100</sup> Higher blood cytokine levels due to cytokine release from a larger  
579 number of infected monocytes and from macrophages in FIP lesions could account  
580 for the more pronounced proliferation and generalised activation of macrophages in  
581 hemolymphatic tissues observed in cats with FIP.<sup>99</sup>

582 Based on the fact that FECV can also replicate at least briefly in monocytes, it was  
583 recently suggested that monocytes, rather than the intestinal epithelial cells, might be  
584 the cells in which the FECV-FIPV mutations occur.<sup>133</sup> Given the high mutation rate of  
585 the virus, this would allow positive selection for macrophage tropism and progressive

viral adaptation to replication in monocytes/macrophages.<sup>133,149</sup> It would also suggest that viral clearance from the blood and even the intestine might not prevent recurrent viremia and possibly even the development of FIP, since persistently infected, healthy, non-viremic FECV carriers were found to bear virus in tissue macrophages, i.e. in sinus macrophages in mesenteric lymph nodes and in pulmonary intravascular macrophages (PIM).<sup>100</sup> This indicates that macrophages in the intestine take up the virus from enterocytes and carry it to the regional lymph nodes and eventually in the blood. Viral RNA was also detected in the liver where the virus most likely infects Kupffer cells (KC).<sup>100,121</sup> Both PIM and KC phagocytose particles from the blood, but could also replicate and release virus into the circulation or transmit it to monocytes. If the mutation and transformation of FECV to FIPV can take place in these macrophages, this could result in FIP at any time post initial infection.<sup>133</sup>

#### *Activation of FIPV-infected monocytes*

The morphological hallmark and initiating lesion of FIP is a granulomatous phlebitis and periphlebitis that is mediated by highly activated monocytes, most likely during a phase of high-level monocyte-associated viremia with substantial viral replication.<sup>84,97,145</sup> Studies on natural cases have shown that the phlebitis develops through direct interaction between monocytes and activated endothelial cells. The monocytes strongly express cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , and adhesion molecules, such as CD18, that allow their interaction with activated endothelial cells, and express enzymes, such as matrix metalloproteinase-9, which dissolve the vascular basement membrane at sites of monocyte emigration; the endothelial cells appear systemically activated and the restrictive distribution of vascular lesions, i.e. veins and in selected organs, is likely a consequence of selective responsiveness of

611 the endothelium.<sup>97</sup> The observed simultaneous, generalised activation of both  
612 vascular endothelial cells and macrophages in hemolymphatic tissues could be  
613 mediated by activated monocytes alone, provided they release sufficient amounts of  
614 cytokines.<sup>97,99</sup> The latter appears likely, considering also that cats with FIP show  
615 increased VEGF transcription in (virus infected) monocytes and increased serum  
616 VEGF levels.<sup>155</sup> Furthermore, peritoneal exudate cells (PEC) of cats with FIP exhibit  
617 high TNF- $\alpha$  mRNA levels and were previously shown to release IL-1 $\beta$  and IL-6, and  
618 even alveolar macrophages collected by bronchoalveolar lavage from FIP cats show  
619 significant upregulation of TNF- $\alpha$ , GM-CSF, granulocyte (G)-CSF, IL-6 and other B  
620 cell differentiation factors, all suggesting strong generalised monocyte/macrophage  
621 activation in response to FIPV.<sup>59,60,150,152,153</sup>

622 What ultimately triggers the fulminant monocyte activation in infected cats is not yet  
623 known. However, FIPV infection of the monocytes is apparently an essential  
624 prerequisite, which was recently shown *in vitro* in isolated feline monocytes and  
625 macrophages. FIPV rapidly induced activation of the p38 mitogen-activated protein  
626 kinase (MAPK), which directly regulates the expression of proinflammatory cytokines  
627 via phosphorylation of a range of signaling molecules, in PBMC, likely early during  
628 entry and, though less intensely, between 6 and 12 hpi, when virus is being  
629 produced.<sup>38,104,137</sup> This was associated with the induction of TNF- $\alpha$  and IL-1 $\beta$ , but not  
630 IL-6 production, as demonstrated in the PBMC supernatant at 24 hpi.<sup>137</sup> VEGF  
631 transcription was shown to be significantly upregulated at 48 hpi in isolated feline  
632 monocytes and alveolar macrophages, and feline alveolar macrophages showed  
633 increased TNF- $\alpha$  production at 48 and 72 hpi, all exclusively in association with viral  
634 replication.<sup>155</sup> A similar increase was seen for G-CSF and GM-CSF transcription at  
635 72 hpi; however, upregulation only became significant and TNF- $\alpha$  and VEGF levels

were further increased, when cells were inoculated with virus in combination with an antibody against the FCoV S protein that is known to induce ADE.<sup>147,152,153,155</sup>

### *The immune system in FIP and FCoV infection*

With the aim to identify the role of the immune system in the pathogenesis of FIP, the blood cytokine transcription (IL-4, IL-6, IL-10, IL-12 p40, IL-18, IFN- $\gamma$ , TNF- $\alpha$ ) was monitored in experimentally infected animals. A first study reported an initial mild increase in IL-6 and IFN- $\gamma$  transcription in PBMC that correlated with transient pyrexia, followed by a drop of all other examined cytokines and IFN- $\gamma$ , possibly as a consequence of the lymphopenia that developed simultaneously.<sup>63</sup> A second study demonstrated TNF- $\alpha$  upregulation during the development of FIP.<sup>103</sup> Another group then screened serum VEGF levels by ELISA and found an increase in association with body effusions.<sup>155</sup> In natural FIP cases, at the time of death, the blood showed very high interindividual variation in cytokine mRNA levels; however, IFN- $\gamma$  mRNA was generally scarce or absent.<sup>53</sup> Blood IFN- $\gamma$  levels of FIP cats were then found to be similar to those of healthy carriers, but high IFN- $\gamma$  concentrations were present in effusions.<sup>56</sup> These were considered a likely consequence of the observed IFN- $\gamma$  transcription within lesions.<sup>16</sup> Believed to be released by the T cells in the lesions, IFN- $\gamma$  could be responsible for macrophage attraction and local activation, which would also enhance Fc receptor expression on their surface and thereby virus uptake and replication.<sup>16</sup> In light of the above results, the generally variable pathological changes and in particular the apparent multiphasic nature of the disease, it is possible that the fulminant monocyte activation, which is essential for the development of FIP vasculitis, does only occur as brief bouts, followed by a phase in which self-sustained granulomatous lesions develop. Such cytokine peaks might be

missed when PBMC or whole blood are sampled on a regular basis or immediately prior to death.

FCoV infection of cats, regardless of the development of FIP, initiates a humoral immune response, as evident by the development of antibody titres and, morphologically, the formation of secondary follicles in lymphatic tissues, and by the presence of FCoV-specific antibodies within plasma cells both in lymphatic tissues and in older FIP lesions.<sup>41,92,94,96,99,164</sup> Some cats can eliminate the infection and then become reinfected, either with a different or the same virus strain, but tend not to develop FIP as a consequence of this.<sup>4,5,49</sup> It is assumed that protective immunity is mainly cell mediated, and there is evidence that the development of high anti-S titers and a high S:M antibody ratio leads to virus clearance.<sup>60,131</sup> However, in many animals, the antibodies that are formed, and in clinical FIP often with very high titres, are not able to eliminate the virus and/or the infected cells. In contrast, experimental studies provided evidence that ADE plays a role in FIP.<sup>122,127,170</sup> FIPV ADE is based on the enhanced uptake of virus and anti-S antibody by macrophages/monocytes via the Fc receptor and can occur *in vivo* when immunized cats are infected with FIPV of the same serotype.<sup>29,113,151</sup> It has however only been seen with some highly virulent FIPV and appears to be of less relevance in the natural disease.<sup>2,131</sup> While ADE could explain the rapid spread of infection in the monocyte/macrophage population, it would not explain why the infected cells are not eliminated by the immune system. The latter can be explained by recent *in vitro* studies. In the presence of specific antibodies, FCoV infected isolated feline monocytes rapidly internalize the viral glycoprotein that is expressed on the surface as antigen-antibody complexes.<sup>39,40</sup> The internalization is mediated by the S and M proteins and, interestingly, does occur with both FIPV and FECV. FIPV also inhibits the complement mediated lysis of

686 infected cells, even if they express viral antigen on their surface.<sup>30</sup> This process is  
687 independent of the accessory 3 and 7 proteins, but the underlying mechanism has  
688 not yet been identified.<sup>30</sup> Nonetheless, the internalization of FCoV proteins by  
689 infected cells may play a role in the pathogenesis of FIP, since the lack of an immune  
690 response that eliminates infected cells will allow more pronounced virus production  
691 and/or a quiescent infection state.<sup>39</sup> This phenomenon could also allow the virus to  
692 persist in tissue macrophages.<sup>100</sup>

693 As mentioned, there are marked differences between the composition and activity of  
694 the hemolymphatic tissues in FCoV-infected cats with and without FIP. Whereas  
695 healthy carriers exhibit distinct lymphoid hyperplasia with lymphocyte proliferation,  
696 FIP cats show depletion which is morphologically reflected in the often markedly  
697 depleted, though mainly secondary, lymphatic follicles, the depletion of T cell zones,  
698 and in particular the generally marked thymus atrophy.<sup>36,94,96</sup> This is mainly a  
699 consequence of lymphocyte apoptosis, and TNF- $\alpha$  expression by lymphocytes, in  
700 particular in lymphatic tissues with FIP lesions, might be the underlying  
701 mechanism.<sup>33,66,96</sup> The findings are matched by the marked blood lymphopenia  
702 observed in the terminal stage of FIP and a persistent drop in circulating CD4+ and  
703 CD8+ T cell numbers as well as an increased rate of apoptosis in PBMC in the  
704 course of the disease.<sup>33,36,150</sup> Among PBMC, mainly CD8+ cells were shown to be  
705 affected by apoptosis, and PEC and PBMC of cats with FIP seem to produce the  
706 necessary TNF- $\alpha$  to mediate this.<sup>150</sup> An *in vivo* study on experimentally infected cats  
707 confirms this finding, as it demonstrated increased TNF- $\alpha$  transcription in PBMC of  
708 cats that developed FIP, whereas animals that remained healthy had low TNF- $\alpha$  and  
709 high IFN- $\gamma$  mRNA levels.<sup>103</sup> High IFN- $\gamma$  transcription, together with an increase in IL-  
710 1 $\beta$ , was also seen in the blood of naturally infected healthy carriers regardless of

antibody titres and virus shedding, prior to the occurrence of FIP cases in the catteries. This was considered a consequence of an increase in CD8<sup>+</sup> T cells and coincided with the release of acute phase proteins, indicating that cytokine production by PBMC contributes to the protection of FCoV infected cats against FIP.<sup>53</sup>

Despite the obvious functional differences in the T and B cell component, FCoV infection is associated with proliferation and activation of monocytes/macrophages and their precursors in hemolymphatic tissues of infected cats and, to a higher degree, cats with FIP.<sup>96</sup> In an attempt to identify the underlying mechanism, hemolymphatic tissues of FCoV infected cats with and without FIP were assessed for the transcription of cytokines that mediate macrophage activation.<sup>99</sup> SPF cats served to establish constitutive transcription levels, which highlighted the general flaw of any such “global expression” study; the high variability in individual transcription levels even in gender and age matched SPF animals, let alone a group as heterogeneous as naturally infected cats with FIP with regard to age, gender, disease type and stage.<sup>53,99</sup> Nonetheless, some relevant differences in the cytokine transcription were identified. Naturally infected healthy cats exhibited significantly higher IL-10 transcription levels in the spleen and lower IL-6, G- and M-CSF levels in mesenteric lymph nodes than cats with FIP, whereas FIP was associated with significantly lower IL-12 p40 mRNA levels in lymphatic tissues.<sup>99</sup> A similar trend of reduced IL-12 p40 transcription was found in lesioned vs. virus-free mesenteric lymph nodes of cats with FIP and in brains with FIP lesions.<sup>33,51</sup> This indicates that an effective immune response together with an IL-10 mediated limitation of macrophage activation and increased cellular cytotoxicity allows infected cats to limit the viral infection and remain healthy, whereas a lack of IL-12 inhibits an effective immune response and

allows monocyte/macrophage activation and ultimately FIP, probably as a consequence of impaired T cell-mediated macrophage activation.<sup>99</sup> The fact that cytokines produced by macrophages and known to activate these and induce their proliferation (such as G-, M- and GM-CSF, IL-6, TNF- $\alpha$ ) were not or not significantly upregulated in these tissues despite the presence of abundant activated macrophages was considered as further evidence of a systemic effect, i.e. the release of the relevant cytokines by infected, activated monocytes.<sup>99</sup>

Several attempts have been made to match the pathogenesis of FIP with known immunopathogenic mechanisms. For example, FIP has long been considered an immune complex-mediated type III hypersensitivity disease, since fibrinogen and C3 was demonstrated cell free and viral antigen, IgG and complement within leukocytes in vascular and focal granulomatous-necrotizing lesions. Also, cats with FIP exhibit FCoV-specific immune complexes in blood and glomerula and show high  $\gamma$ -globulin and C3 serum levels.<sup>85,86,117,122,169,170</sup> However, circulating FCoV-specific immune complexes are not only found in diseased animals, but can also be detected, at least transiently, in infected cats that remain healthy.<sup>92,109</sup> Also, the typical FIP vasculitis does not show features of immune complex vasculitis.<sup>97</sup> While this does not confirm a type III reaction as the essential pathogenic mechanism, it does not exclude its contribution to the disease, for example in its acceleration (see above). Other authors have considered a type IV hypersensitivity reaction as the basis for the development of the granulomatous lesions, due to the dominance of CD4+ cells in the otherwise macrophage dominated lesions.<sup>16,118</sup> In any case, the available data provide definite evidence that the immunity to FIP is cell mediated and it has been postulated that it requires viral persistence.<sup>131</sup> Furthermore, there is evidence from both experimental



and natural infections that an effective early T cell response to FCoV is essential for the prevention of FIP, since it appears to ensure the limitation of viral replication.<sup>36,119</sup> There has been a lot of speculation why cats develop the dry, the wet or a mixed form of FIP, and it is widely agreed that a strong humoral together with a very weak cellular immunity leads to the former, with lesser effusion with increasing cellular immunity.<sup>131</sup> However, a recent study provided evidence that also the effusions are initiated by FIPV-infected monocytes/macrophages, since the latter have been shown to produce VEGF.<sup>155</sup> VEGF is a very strong mediator of vascular permeability and does cause hyperpermeability also of feline vascular endothelial cells.<sup>43,155</sup> Also, serum VEGF levels were seen to correlate with the quantity of body effusions.<sup>155</sup> These findings do not necessarily contradict the existing theories, but are very much in line with all other data that render the infected, activated monocyte the key mediator of the disease. Several studies have provided data that indicate individual differences in susceptibility to FCoV infection in general and to the effect of FIPV on monocytes. Molecular studies found evidence that individual cats can be entirely resistant to FCoV infection and that the monocytes of some cats can be completely resistant to FCoV, or to FECV alone, while the monocytes of some cats are more prone to productive FCoV infection than those of others.<sup>4,5,38,157</sup> Finally, there appear to be differences in FIPV serotype I internalization in individual cats, suggesting that its receptor is expressed differentially between cats.<sup>160</sup> At present, the knowledge is still lacking as to the reason for the individual differences and the key event that blocks the capability of monocytes to inhibit virus production.

## **Diagnosis of FIP**

The post mortem diagnosis of FIP relies on a combination of gross and histological examination, in combination with the demonstration of viral antigen in the lesions.<sup>57</sup> In contrast, the non-invasive ante-mortem diagnosis of FIP still remains a challenge, especially in the dry form of the disease.<sup>71,95,131</sup> A combination of indirect and/or direct virus detection with evaluation of blood hematological and chemical parameter as well as medical history and clinical symptoms, the so-called FIP algorithm, is so far the best predictor of disease.<sup>6</sup>

### *Host blood parameters*

Common blood alterations include lymphopenia, mild to moderate regenerative anemia, hyperproteinemia, and hypergammaglobulinemia. Other laboratory parameters, such as liver enzymes, bilirubin, urea and creatinine might be helpful, but high values merely reflect organ damage, which is most likely a consequence of FIP lesions.<sup>6</sup>

More than the single parameters, the albumin to globulin ratio (A:G ratio) has a high diagnostic value, and at values above 0.8, FIP is extremely unlikely.<sup>6,70</sup> A more recent retrospective study evaluating the A:G ratio showed a very poor positive predictive value (PPV) even for a cut-off value of 0.6. However, the negative predictive value (NPV) was 100% and 99% for an A:G ratio of <0.8 and <0.6%, respectively.<sup>88</sup>

Recent studies have focused on the diagnostic value of an acute phase protein, alpha-1 acid glycoprotein (AGP). Serum levels are highly elevated in cats with FIP (> 3 mg/ml), but are also high in other inflammatory conditions or neoplastic diseases, such as lymphoma.<sup>25,31,141</sup> Furthermore, AGP levels may also rise in asymptomatic FCoV carriers, especially from households with endemic infection.<sup>54</sup> However, when

810 interpreted alongside pre-tests, i.e. epidemiological factors, clinical information and  
811 FCoV serology, moderate AGP increases are useful discrimination parameters when  
812 the probability of FIP is high, whereas with low FIP probability, only very high AGP  
813 levels support the diagnosis of FIP.<sup>120</sup> A recent retrospective study found complete  
814 concordance between AGP levels and immunohistology in challenging diagnostic  
815 cases.<sup>54</sup>

### 817 *Analysis of effusions*

818 The presence of effusions facilitates the diagnosis, since tests on effusions have a  
819 higher diagnostic value than blood tests.<sup>6,70</sup> FIP effusions typically have a very high  
820 protein content (>35 g/l), but a low cellularity (<5000 nucleated cells/ml) with a  
821 dominance of macrophages and neutrophils. When sufficient cells are present, the  
822 demonstration of viral antigen in macrophages confirms the diagnosis with a very  
823 high PPV.<sup>22,70,71</sup>

824 The Rivalta test, commonly used to differentiate between FIP effusions and effusions  
825 due to other diseases, is not very specific. The high protein content, including fibrin  
826 and inflammatory mediators, in FIP effusions normally induces a positive reaction.  
827 However, a recent study on a large cohort of cats with effusions has shown that,  
828 while it has a high NPV, this test has a lower sensitivity, specificity, and PPV than  
829 previously reported.<sup>47,71</sup>

830 A:G ratio can also be measured in effusions with high PPV if the ratio is <0.4, and  
831 with high NPV if the ratio is >0.8.<sup>142</sup> Also, very good correlation exists between AGP  
832 values in effusions and serum.<sup>14</sup> The demonstration of FCoV-specific antibodies in  
833 the effusions is only meaningful when the titre is high ( $\geq 1:1600$ ), whereas the  
834 absence of antibodies has a good NPV.<sup>70</sup>

835

836 *Indirect virus detection: serology*

837 Serology, based on the detection of FCoV antibody titres by a range of methods  
838 (immunofluorescence, ELISA, rapid immunomigration) is widely used also  
839 commercially to assist in the diagnosis of FIP and for quarantine purposes. The tests  
840 are applied to blood and effusions, and apparent false negative results are a known  
841 problem. A recent study addressed this issue and showed a correlation of lower  
842 antibody levels in samples containing higher amounts of virus, as shown by qRT-  
843 PCR.<sup>110</sup> It was hypothesized that these false negative results were due to antibody  
844 binding to virus in the sample instead of the virus in the serological tests.<sup>110</sup>  
845 Also, a high percentage of healthy FCoV carriers are antibody-positive, of which only  
846 a small percentage develops FIP.<sup>1,146</sup> Very high titres ( $\geq 1:1600$ ) in combination with  
847 pre-tests that suggest FIP indicate an increased likelihood of FIP, unless obtained  
848 from animals in an endemic environment, such as multiple-cat households.<sup>70,130</sup> It  
849 should also be noted that different methodological approaches and even different  
850 laboratories might yield different results from the same sample, depending on the  
851 antigen used.<sup>71</sup>

852 Serology is generally considered a useful tool for the screening and management of  
853 catteries and quarantine purposes, in particular since antibody titres are correlated to  
854 shedding intensity and frequency.<sup>41</sup>

855 The detection of circulating antigen-antibody complexes, for example by a  
856 competitive ELISA on serum, was shown to have a PPV of 67% and a NPV was  
857 84%.<sup>70,85</sup> However, healthy FCoV carriers can also show circulating immune  
858 complexes.<sup>109</sup>

859

## Direct virus detection

Immunohistology has been used for two decades to detect FCoV antigen in lesions and has a very high PPV.<sup>22,92,118,156</sup> It is therefore considered the gold standard and by many diagnostic pathologists an essential component of the definite diagnosis of FIP, in particular in histologically inconclusive cases.<sup>41,57,131</sup> For the definite ante mortem diagnosis, optimally, surgical biopsies of granulomatous lesions are used (Fig. 7), while random Tru-cut biopsies or fine needle aspirates are often not helpful.<sup>55</sup> The demonstration of FCoV antigen in macrophages in effusions, as mentioned above, is an alternative, non-invasive tool for the *intra vitam* diagnosis of FIP. A positive result is highly predictive of FIP, whereas a negative result does not exclude FIP.<sup>22,70</sup> The latter is due to both the low cellularity of the effusions and the relatively low sensitivity of the method, which can only detect heavily virus laden cells. In the authors' experience, based on the parallel staining of cytological preparations and formalin-fixed, paraffin embedded cell pellets prepared from the effusions (minimum 1 ml), more reliable results can be obtained from the concentrated cell preparations in the pellets (Fig. 8).

RT-PCR, especially real-time RT-PCR, is a sensitive method to detect virus RNA in different samples, such as feces, blood, effusions, and tissues of FCoV-infected cats and those with FIP, however, these cannot differentiate between the pathotypes.<sup>45,65,77,102</sup> The detection of FCoV RNA in the feces is mainly used for management purposes in catteries, i.e. to determine the kinetics of viral shedding.<sup>4,41</sup> FCoV is known to spread systemically with infection, regardless of the development or presence of clinical signs of FIP; therefore, diagnostic tests that identify viremia can only be used to support other tests towards a diagnosis of FIP in cats with

relevant clinical features.<sup>41,62,109</sup> The detection of virus in effusions, however, has proven to have a high PPV, but a negative result does not exclude FIP.<sup>52,159</sup> A recent study identified two alternative amino acid differences in the putative fusion peptide of the FIPV S protein in FECV and FIPV and confirmed that together these two substitutions distinguish FIPV from FECV in >95% of cases.<sup>28</sup> Although it cannot be excluded, due to the quasispecies nature of FCoV, that other mutation patterns could lead to disease, so far, this is the most promising potential diagnostic tool that involves the direct virus detection.<sup>11,42,64,102</sup> Nevertheless, as these alterations are not present in the virus shed by cats with clinical FIP, any routinely employed diagnostic test based on this result will need to be sufficiently sensitive. Also, as a commercial test, a protocol would be preferable that could directly detect these mutations without the need of a further, time-consuming sequencing step.

A recent publication takes previous attempts to correlate FIPV replication in monocytes with FIP further and suggests a new methodological approach for the robust simultaneous detection of virus replication and viral load, by a real time PCR based on primer-probe energy transfer.<sup>24,84,145</sup> Rather than a tool to identify infected cats, the authors proposed this method for the identification of persistent shedders and thereby the potential sources of emerging FIP variants. Since the test was also able to reliably detect virus replication in FIP effusions, it might be useful for the confirmation of FIP at least in the wet form, provided the results are confirmed on larger case cohorts.<sup>84</sup>

With increasing knowledge of the pathophysiological mechanisms that drive the virus-host interaction in FCoV infection and with the constant improvement of molecular techniques, there is reasonable hope that in the near future, the diagnostic

tools for the diagnosis of FIP can be refined to specifically detect FIPV and to integrate the assessment of more host response parameters tailored to FIPV.

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1418

## Figure Legends

**Fig. 1.** Gross FIP lesions. a. Cat with wet FIP, exhibiting a serofibrinous and granulomatous serositis and granulomatous lesions in the liver (arrows). b-f. Cats with dry FIP. b. Enlarged mesenteric lymph node with granulomatous inflammation. c. Jejunum with multiple granulomas in the serosa. d. Jejunum with small subserosal granulomatous lesions that follow the veins (phlebitis and/or periphlebitis; arrow). e. Kidney with granulomatous phlebitis and periphlebitis of a Vena capsularis. f. Cat with dry FIP and multifocal granulomatous phlebitis and periphlebitis of a cortical leptomeningeal vein (arrow). All lesions were confirmed by subsequent histological examination and immunohistological demonstration of FCoV antigen within the lesions.

**Fig. 2.** Distribution of FIP lesions in cats that had undergone a thorough post mortem and histological examination for the diagnosis of FIP, including the histological examination of all major organs and tissues and confirmation of the disease by the immunohistological demonstration of FCoV antigen. N=77 (<sup>1</sup>N = 54; <sup>2</sup>N = 86).

**Fig. 3.** Natural FIP case with thoracic effusion and fibrinous and granulomatous pleuritis. Pleura with inflammatory processes of variable duration. a) Overview with dense basal layer of plasma cells (black arrowheads), overlain by layer of granulation tissue with new vessels (arrows) and embedded macrophage dominated infiltrates (white arrowheads) and surface layer of fibrin (\*) with embedded inflammatory cells. HE stain; Bar = 50µm. b) In another area, the basal plasma cell layer (black arrowhead) is overlain by a loose granulation tissue with new vessels (arrows) and

occasional fibroblasts (white arrowheads). HE stain; Bar = 20µm. c) Granuloma in the granulation tissue. FCoV antigen is present within several macrophages in the granuloma. Horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70, Meyer's hematoxylin counterstain.<sup>92</sup> Bar = 20µm.

**Fig. 4.** Natural FIP case with thoracic effusion and with fibrinous and granulomatous pleuritis (see Fig. 3). Diaphragm, exhibiting a chronic plasma cell dominated (black arrowheads) diffuse pleuritis with new vessel formation (arrows) consistent with granulation tissue formation. Towards the surface, an infiltrate of macrophages and neutrophils is observed (white arrowheads). Occasional macrophages are found that express FCoV antigen (inset). HE stain and horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70, Meyer's hematoxylin counterstain (inset) .<sup>92</sup> Bars = 20µm (inset: Bar = 10µm).

**Fig. 5.** Natural FIP case, dry form. Kidney, cortex with multiple FIP lesions. a) Stellate vein (SV) with fibrinoid necrosis (arrows) and granulomatous vasculitis, partly occluded by leukocytes (arrowhead). HE stain; Bar = 50µm. b) Closer view of a), highlighting the necrosis of the vessel wall (arrows) and the infiltrate, dominated by often degenerate macrophages. There are also focal plasma cell aggregates (arrowhead) immediately outside the vascular wall. HE stain; Bar = 20µm. c) Closer view of b). FCoV antigen is present within monocytes in the vascular lumen (arrowheads) and in the vasculitis (arrow). Horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70, Meyer's hematoxylin counterstain.<sup>92</sup> Bar = 10µm. d) Stellate vein distant from the vein in a)-c), exhibiting two focal perivascular plasma

1468 cell accumulations and activated endothelial cells (arrowheads). Bar = HE stain;  
1469 20µm.

1470

1471 **Fig. 6.** SPF cat euthanized without clinical signs and post mortem changes  
1472 consistent with FIP. The animal had been housed for 30 weeks with animals dying  
1473 from FIP and had shown a clinical episode of CNS symptoms and ocular changes  
1474 consistent with FIP. Brain, medulla oblongata. a) Mononuclear perivascular infiltrates  
1475 (arrows) in the white matter and diffuse infiltrates in the leptomeninges (\*). HE stain; Bar  
1476 = 50µm. b) The infiltrate is dominated by CD45R-positive B cells. Avidin biotin  
1477 complex peroxidase method (rat anti-mouse CD45R, clone B220/Ly5,  
1478 Papanicolaou's hematoxylin counterstain.<sup>92</sup> Bar = 50µm. c) The perivascular infiltrate  
1479 (arrow) as well as the leptomeningeal infiltrate contains plasma cells with FCoV-  
1480 specific antibodies. Peroxidase anti-peroxidase method (DF-2 FIPV suspension,  
1481 followed by mouse anti FCoV (clone FCoV3-70), Papanicolaou's hematoxylin  
1482 counterstain.<sup>92</sup> Bar = 10µm.

1483

1484 **Fig. 7.** Natural FIP case, dry form. Mesenteric lymph node biopsy. a) Granulomatous  
1485 lesions are present in the serosa (\*) and occasionally within the lymph node in  
1486 association with the cortical sinuses (arrow). HE stain; Bar = 50µm. b) Viral antigen is  
1487 expressed by macrophages in the serosal lesions and in parenchymal lesions (inset:  
1488 higher magnification of \*). Horseradish peroxidase method (mouse anti-CoV, clone  
1489 FCoV3-70, Meyer's hematoxylin counterstain.<sup>92</sup> Bar = 50µm. Inset: Bar = 10µm.

1490

1491 **Fig. 8.** Natural FIP case, wet form with abundant abdominal effusion. a) Cytological  
1492 specimen (smear) from the effusion, comprised of macrophages/mesothelial cells

1493 (arrow) and neutrophils (arrowhead). May-Grünwald-Giemsa stain. b) Macrophages  
1494 in the smear express viral antigen. c) Macrophages in a formalin fixed and paraffin  
1495 embedded cell pellet express abundant (arrow) to small amounts (arrowhead) of viral  
1496 antigen. Horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70, Meyer's  
1497 hematoxylin counterstain.<sup>92</sup> Bars = 10µm.